DISCOVERY AND GENETIC ASSESSMENT OF WILD BOTTLE GOURD [LAGENARIA SICERARIA (MOL.) STANDLEY; CUCURBITACEAE] FROM ZIMBABWE[1]

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Decker-Walters, Deena S., Mary Wilkins-Ellert *(The Cucurbit Network, P.O. Box 560483, Miami, FL 33256; cucurbitnetwork@netscape.net),* **Sang-Min Chung and Jack E. Staub** *(USDA/ARS, Vegetable Crops Research Unit, Department of Horticulture, 1575 Linden Dr., University of Wisconsin, Madison, WI 53706).* DISCOVERY AND GENETIC ASSESSMENT OF WILD BOTTLE GOURD [*LAGENARIA SICERARIA* (MOL.) STANDLEY; CUCURBITACEAE] FROM ZIMBABWE. *Economic Botany 58(4):501–508, 2004. Bottle gourd [*Lagenaria siceraria *(Mol.) Standley] is an edible, medicinal, and otherwise utilitarian domesticated cucurbit with an ancient pantropical distribution. This African native reached Asia and the Americas 9000 years ago, probably as a wild species whose fruits had floated across the seas. Independent domestications from wild populations are believed to have occurred in both the Old and New Worlds. However, few wild populations of* L. siceraria *have been found during recorded history and none has been verified or studied in detail. In 1992, Mary Wilkins-Ellert discovered an unusual free-living plant of* Lagenaria *in a remote region of southeastern Zimbabwe. Her morphological observations during several plantings of the collected seed, as well as results from two genetic analyses (random amplified polymorphic DNA and chloroplast sequencing), indicate that the Zimbabwe collection is part of a genetically distinct and wild lineage of* L. siceraria.

Key Words: Bottle gourd, *Lagenaria siceraria, Lagenaria sphaerica,* RAPD, cpDNA sequencing.

Bottle gourd [*Lagenaria siceraria* (Mol.) Standley; Cucurbitaceae] was one of the first plant species to be domesticated for human use, providing food, medicine, and a wide variety of utensils and instruments made from the large, hard-shelled fruit. The five wild congeners are native to the northern half of Africa, with *L. sphaerica* (Sond.) Naud. and *L. breviflora* (Benth.) G. Roberty occurring as far south as South Africa and Zimbabwe, respectively (Jeffrey 1967). Thus, the ancestral home of *L. siceraria* is believed to be Africa even though the oldest African archaeological remains date to only about 2000 B.C. in Egypt (Schweinfurth 1844) and Zambia (Fagan 1970). In contast, archaeological seeds and rind indicate that bottle gourd had reached Asia and the New World by 9000–10 000 years ago (Cutler and Whitaker 1961; Gorman 1969; MacNeish et al. 1970), probably as a wild species whose fruits had floated across the seas (Heiser 1973).

In spite of the ancient and modern pantropical distributions of domesticated bottle gourd (Heiser 1979; Richardson 1972) and the occasional escapes from cultivation (DeCandolle 1967), few potentially indigenous wild populations have been found during recorded history. In 1886, DeCandolle (1967) discussed the earliest documented sightings of purportedly wild bottle gourd. He considered most convincing the written reports of populations from India and Ethiopia. In *Flora Indica,* Roxburgh (1832) mentioned "A wild bitter variety called *Tita Laoo* . . . ," but more recent floras (e.g., Chakravarty 1959; Saldanha and Eswar Rao 1984) describe bottle gourd only as a cultivated or possibly escaped plant on the Indian subcontinent. In *Flora of Ethiopia and Eritrea,* Jeffrey (1995) described bottle gourd as occurring in ". . . bushland and grassland . . . ," confirming earlier sightings of apparently indigenous plants in this region of northeastern Africa (DeCandolle

[1] Received 29 November 2003; accepted 20 January 2004.

1967). Other 20th-century evidence of wild populations includes the listing of four herbarium specimens of *L. siceraria* collected from regions of South Africa where escapes from historical cultivation would be unlikely according to Meeuse (1962). However, in *Flora Zambesiaca* (F.Z.), Jeffrey (1978) stated that *L. siceraria* was "... perhaps nowhere truly wild in the F.Z. area ...," with the flora's coverage including Zimbabwe, Zambia, Malawi, Botswana, and Mozambique. None of the purportedly wild bottle gourd populations has been studied in morphological or genetic detail.

As one of the earliest domesticated plants, *L. siceraria* is pivotal to issues concerning the mechanisms and cultural parameters of the earliest human transitions to plant domestication. The discovery and genetic evaluation of wild populations of this species could also impact various competing hypotheses concerning the domestication (e.g., single vs. multiple events) and earliest dispersals (e.g., by humans, oceanic transport, or both) of bottle gourd specifically. As discussed in Doebley (1989), molecular markers [e.g., isozymes, random amplified polymorphic DNA (RAPDs), single nucleotide polymorphisms (SNPs), etc.] can be used to determine whether wild populations of a domesticated species represent recent or ancient escapes from cultivation (if so, they should exhibit a subset of the variation present in the domesticate) or if they represent the wild lineage from which the domesticate was first selected (in which case, the domesticate should exhibit a subset of the variation present in the wild ancestor). We describe herein the discovery and genetic assessment of a wild population of *Lagenaria,* and discuss how our findings relate to the history of bottle gourd domestication.

Discovery of an Unusual Wild *Lagenaria* Population

On March 18, 1992, the second author, Mary Wilkins-Ellert, discovered gourd fruits hanging high up in a tree in mopane [*Colophospermum mopane* (J. Kirk ex Benth.) J. Leonard; Fabaceae] woodland next to a dry stream bed in the Gonarezhou National Park on Zimbabwe's eastern border with Mozambique. There were no villages or human habitations in this remote area (ca. 300 m ASL, 21° 56′ S, 31° 29′ E).

The cucurbit vine(s) were dead, but numerous spherical gourds measuring 8.5 cm wide × 9.0 cm long were attached to the dried branches. The fruits were light green with pale blotches, which were mostly on the lower half of the fruit (Fig. 1). The overall visual appearance of the fruits was indicative of a wild species such as *L. sphaerica* or *L. breviflora,* both of which are native to Zimbabwe. Seed set quantities appeared normal for *Lagenaria* fruits, i.e., most seed coats were full. The rectangular seeds (Fig. 2) had two short protuberances ("ears") at the end distal to the seed scar, which is typical of *L. sphaerica* and *L. siceraria,* but not of *L. breviflora* (Jeffrey 1978). The seeds were the size of those of *L. sphaerica,* except that they were relatively thick (10–13 mm long × 4–5 mm wide × 3 mm thick). Although the seeds were brown as in *L. siceraria* (in contrast to the typical white in *L. sphaerica*), the seed was smooth in its entirety (e.g., lacking hairs or marginal ridges). A smooth seed coat has not been previously documented in species of *Lagenaria* (Jeffrey 1967, 1978).

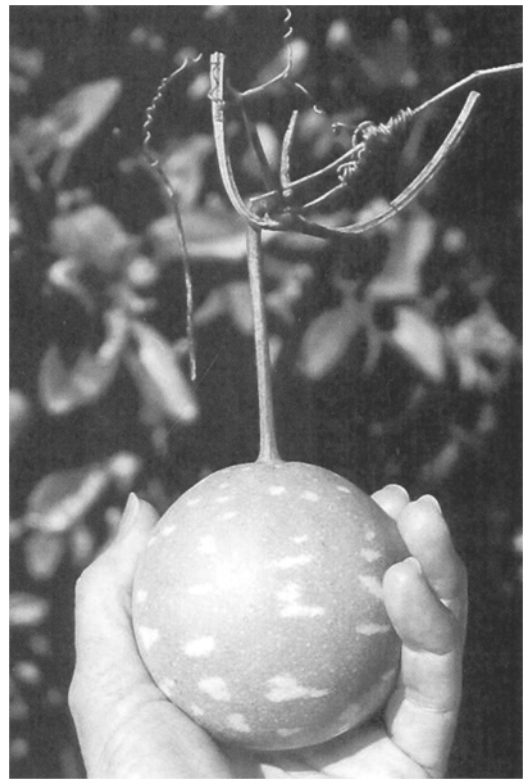

Fig. 1. A second generation fruit produced from seed of the wild *Lagenaria* population discovered in southeastern Zimbabwe.

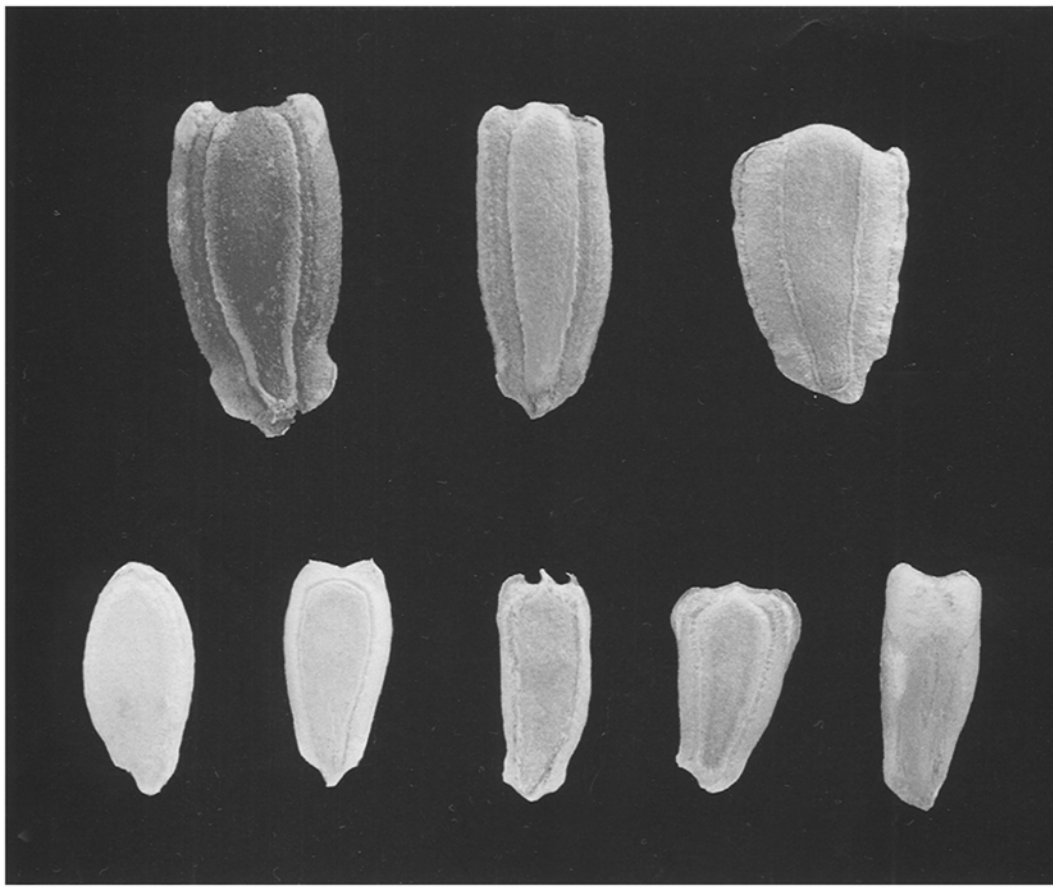

Fig. 2. Top row, left to right: seeds of domesticated *L. siceraria* (#1532, 1522, 1523); bottom row, left to right: seeds of *L. breviflora* (#1524), *L. sphaerica* (#1111, 1525, 1536), wild Zimbabwe collection (#1502). The seeds vary from dark brown (#1532) to tan (#1111, 1525). The seed of #1502 measures 13 mm long.

Overall, the seeds and fruits from Wilkins-Ellert's wild population (hereafter referred to as the Zimbabwe collection) shared characteristics with both *L. sphaerica* and *L. siceraria,* as well as possessed unique traits. Fertile hybrids between these species have been produced (Cogniaux and Harms 1924; Meeuse 1962), and Jeffrey (1967) suggested that such hybrids be looked for in the wild. Consequently, the possibility had to be considered that the plant(s) of the Zimbabwe collection were progeny of a cross between the two species, even though there had been no evidence in the field of the cultivation or other human activity required to supply domesticated *L. siceraria* as a reproductive participant.

Seeds from the Zimbabwe collection were planted by Wilkins-Ellert in a garden plot in Bulawayo, Zimbabwe. Several plantings from original seed were made between 1996 and 2000; in addition, seeds from one of the increased plants were planted to create yet another generation of fruits. For all of the plants grown by Wilkins-Ellert, fruits set through open pollination and normal quantities of full seeds developed, with no evidence of segregation in fruit characters. The fruits were smaller than those of the original collection, measuring ca. 7.4 cm wide × 7.3 cm long. Once dry, the rind was not durable as is typical of *L. siceraria.* Instead, the exocarp became very thin, was easily cracked, and ultimately disintegrated after several years.

The seeds produced from the second and third generation plants exhibited limited variation; most resembled the original collection in that they were brown, relatively thick, and lacked

hairs or ridges. However, at least one fruit produced seeds that were lighter in color, had more pronounced "ears", and exhibited the raised hairy longitudinal ridges characteristic of domesticated *L. siceraria.* This fruit, and corresponding mother plant, are evidence of genetic segregation in seed characteristics.

Aside from the atypical fruit and seed traits, the increased plants exhibited characteristics that were all similar to those of *L. siceraria,* including monoecy, a musky smell, softly tomentose stems and leaves, a fibrous root system, and solitary, night-blooming flowers.

The morphophysiological characters of the Zimbabwe collection indicated that it was probably a previously unknown wild *L. siceraria* (possibly representing the ancestral lineage of the domesticate) or that it was a domesticated bottle gourd escape that at some point had undergone introgression with *L. sphaerica.* Molecular genetic methodologies could be used to examine these and other hypotheses concerning the evolutionary origins of the Zimbabwe collection. Two sources of molecular data that had proved useful in previous studies of bottle gourd were the analysis of fragment size variation of total genomic DNA using RAPD primers (Decker-Walters et al. 2001) and sequencing of the plastid genome to identify SNPs, insertions, deletions, and inversions (Chung et al. 2003; Decker-Walters et al. 2004). RAPD markers, which are variable within *L. siceraria* and typically distinct from this species in *L. sphaerica* (Decker-Walters et al. 2001), were surveyed to determine whether or not the Zimbabwe collection could be an escape of domesticated *L. siceraria* that possessed introgressed genes from *L. sphaerica.* If this were the case, then the RAPD banding patterns of the Zimbabwe collection should exhibit a combination of the differing bands present in both species, as well as a small percentage of unique bands (Huang et al. 2000). However, unique bands could also be an indicator that the Zimbabwe collection is genetically distinct, representing a distinct lineage of *L. sphaerica* or *L. siceraria* (e.g., the wild ancestor of the domesticate) or even a new species, depending on the extent of RAPD differentiation.

Since homology among RAPD bands, particularly for different species, can be questionable (Rieseberg 1996), and results might not point to a definite conclusion concerning the evolutionary history of the Zimbabwe collection, we collected plastid sequence data, which are more conserved and less variable than RAPDs within species (Decker-Walters et al. 2004), to more accurately determine the taxonomic status of the Zimbabwe collection as well as the putative evolutionary relationships among this collection, domesticated *L. siceraria, L. sphaerica,* and *L. breviflora.* For example, we expected the plastid DNA of the Zimbabwe collection to be identical to that of domesticated *L. siceraria* or *L. sphaerica* if one of these taxa represented the maternal ancestor of the collection, or to be distinct if the collection represented a separate lineage (either within one of these species or as a distinct species) with a long, independent evolutionary past. In the latter case, the collection would be deemed to represent a truly wild taxon, although substantial differentiation compared to domesticated *L. siceraria* would suggest that bottle gourd domestication did not directly involve this lineage. Various other interpretations concerning the evolutionary origins of the Zimbabwe collection were possible depending on the nature of the combined RAPD and plastid sequence data.

Materials and Methods

RAPD Analysis. For the RAPD analysis, DNA was extracted from two seeds of the original Zimbabwe collection (TCN accession #1502), and one seed each of three domesticated *L. siceraria* collections [Kenya (#1522), Egypt (#1523), and South Africa (#1532)], four *L. sphaerica* collections [Zimbabwe (#1525, 1536), South Africa (#1111, 1127)], and one *L. breviflora* collection [Zimbabwe (#1524)]. Mary Wilkins-Ellert (MW) collected accessions #1502 (MW 339), 1525 (MW 295), 1536 (MW 442), and 1524 (MW 234). As she was able to carry only small seed samples with her when she fled Zimbabwe for political reasons in 2001, we sacrificed only 1–2 seeds per accession. The remaining accessions were collected or otherwise obtained (some of the domesticated gourds were purchased) by Deena Decker-Walters. Additional comparative material consisted of the 74 accessions of domesticated *L. siceraria* in Decker-Walters et al. (2001), which included several accessions from Zimbabwe and other parts of southern Africa.

RAPD PCR amplifications were carried out according to Decker-Walters et al. (2001) using RAPD primers from Operon Technologies (Al-

ameda, CA). The chosen primers—AB6, AI10, AS14, M12—had exhibited clear banding pattern differences between *L. siceraria* and *L. sphaerica* in a previous study (Decker-Walters et al. 2001). After completion of the PCR, 5 uL of loading dye (0.1% bromophenol blue, 0.1% xylene cyanoll FF, 10% Ficol) was added to each reaction tube. The samples were electrophoresed in 1.6% agarose gels containing 0.5 μg/mL ethidium bromide in 0.5X TBE buffer (0.045 M Tris-borate and 1.0 mM EDTA pH 8.0), for 4 hr at 180 volts. Banding patterns were visualized using a Dark Reader™ transilluminator (Clare Chemical, Denver, CO) that does not use UV light, then captured with a digital camera, and recorded using "Gel Expert" (Nucleotech Corp., San Mateo, CA). Banding patterns were compared by counting the number of bands in the Zimbabwe collection that were shared with each of the three species.

cpDNA Analysis. The accessions used in the RAPD study were also used in the sequencing study, as well as 13 additional landraces of domesticated *L. siceraria* from Africa [Niger (TCN#469), Senegal (#1103), Ethiopia (#1530), South Africa (#1519), Zaire (#1526)], Asia [Iraq (#1527), India (#839), Thailand (#1513), China (#1534)], and the Americas [Honduras (#1029), Peru (#1515), Brazil (#841), Chile (#1367)]. An accession (TCN#1167; GenBank #AY437555) from a comparative analysis of wild and cultivated *Cucurbita pepo* L. (Decker-Walters et al. unpubl. data) was included as an outgroup with which to polarize nucleotide differences in *Lagenaria.*

In the process of collecting data on plastid DNA sequence variation for *Lagenaria* and related genera (Decker-Walters et al. 2004), a few single nucleotide polymorphisms (SNPs) were detected for *L. siceraria.* Here, we report sequence variation among species of *Lagenaria* for the plastid DNA segment labeled ccSSR-4 in Chung et al. (2003), the primers for which are 5′AGGTTCAAATCCTATTGGACGCA3′ (forward) and 5′TTTTGAAAGAAGCTATTCARGAAC3′ (reverse). The sequence, most of which does not occur in *Nicotiana tabacum* (GenBank #Z00044), is approximately 600 bp long in *Lagenaria* and primarily represents the *trnR–atpA* spacer. Positional numbering for the *Lagenaria* SNPs is in the form of the last clearly homologous position (#10577) with *Nicotiana tabacum* plus the number of additional positions (in the 5′ to 3′ direction) in the *Lagenaria* sequences (e.g., 10577+40). *Lagenaria* sequences have been deposited in GenBank (#AY437533–AY437554).

Once the accessions and primers were chosen, DNA was extracted from one seed of each accession according to previous protocols (Chung et al. 2003) with the following modifications. DNA was dissolved with distilled water after the precipitation. Proteins were then re-precipitated and removed again by adding one-third volume of 5 M ammonium acetate. DNAs were then re-precipitated by adding 100% ethanol and subsequently washed twice with 70% ethanol. DNA was quantified using a mini-fluorometer (model TD-360, Turner Designs, Sunnyvale, CA). PCR protocols followed Chung et al. 2003, and the amplified fragments were sequenced according to Chung and Staub (2003). Genetool software (BioTools Inc., Edmonton, Canada) was used to align the sequences.

Phylogenetic analysis of the polymorphisms (e.g., SNPs; see RESULTS) in the aligned sequences was performed with PAUP* 4.0b10 (Swofford 2002), using *Cucurbita pepo* #1167 as the outgroup. Only SNPs in *Lagenaria* were evaluated for tree constuction. An exhaustive search was selected to find minimal tree lengths (i.e., maximum parsimony analysis), and character optimization was performed with the DELTRAN algorithm in PAUP*.

RESULTS

The patterns of band migration on the RAPD gels (Fig. 3) were highly similar for the Zimbabwe collection and the examined accessions of domesticated *L. siceraria.* However, the Zimbabwe samples also exhibited several bands not found in the samples of *L. siceraria, L. sphaerica,* or *L. breviflora.* Band comparisons among the Zimbabwe collection and the three species across all four RAPD systems (including some lightly staining bands not clearly visible in Fig. 3) revealed a total of 42 bands in the Zimbabwe collection, 15 of which were unique to this collection. Also unique to this collection was the absence of four bands (starred in Fig. 3) that were shared by domesticated *L. siceraria* and *L. sphaerica* accessions. The starred band in AI10 is particularly noteworthy in that it was present in all *L. siceraria* and *L. sphaerica* samples examined herein and in Decker-Walters et al. (2001).

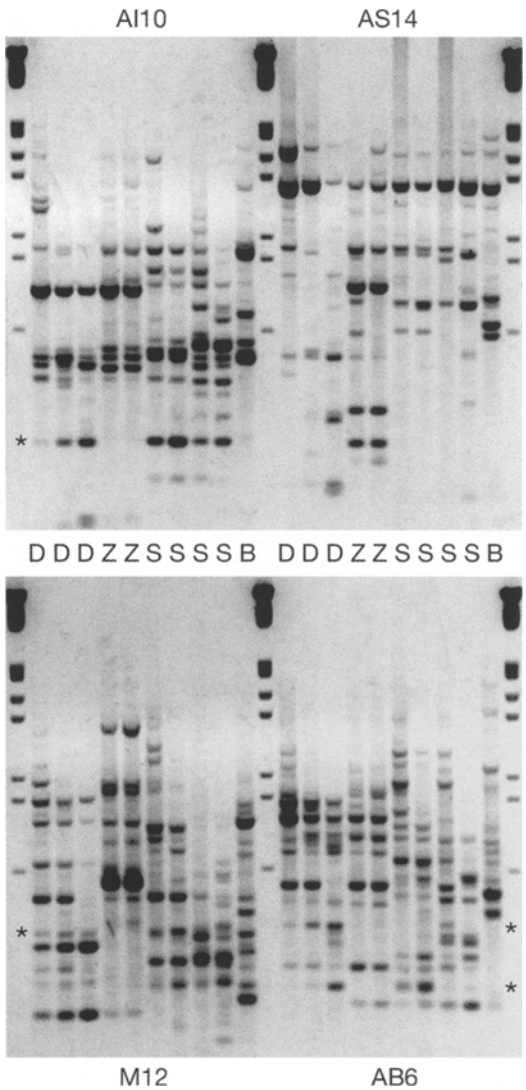

Fig. 3. Banding patterns created by the RAPD primers AI10, AS14, M12, and AB6 for three accessions of domesticated *L. siceraria* (D), two seeds from the Zimbabwe collection (Z), four accessions of *L. sphaerica* (S), and one accession of *L. breviflora* (B). A Lambda DNA *Eco*RI+*Hind*III size ladder is located on either side and in the middle of each panel. Band positions marked with an asterisk represent bands that are present in domesticated *L. siceraria* and *L. sphaerica,* but missing in the Zimbabwe samples.

The domesticated *L. siceraria* samples in Fig. 3 shared 22 bands in common with the Zimbabwe collection, eight of which were not present in *L. sphaerica* or *L. breviflora. Lagenaria sphaerica* shared 17 bands with the Zimbabwe collection. However, only three of those bands were absent in domesticated *L. siceraria.* Similarly, *L. breviflora* shared nine bands with the Zimbabwe collection, only three of which were absent in domesticated *L. siceraria.*

Five SNPs were found in *Lagenaria*—#1 (10577+40), #2 (+41), #3 (+47), #4 (+251), and #5 (+343). Nucleotides found at each SNP site are listed in Table 1. Multiple character states in *L. sphaerica* were found for SNP sites #1 and #2. All accessions of domesticated *L. siceraria* exhibited identical genotypes, which differed from the genotype of the Zimbabwe collection at site #1 (Table 1).

Phylogenetic tree construction yielded a single most parsimonious topology in which *L. sphaerica* and *L. breviflora* uniquely shared a common ancestor and *L. siceraria* and the Zimbabwe collection uniquely shared a common ancestor (Fig. 4). Two character change convergences (either naturally occurring parallelisms or occurrences due to introgression) occurred in *L. sphaerica.* These convergences represent character states in addition to the presence of the ancestral states at SNP sites #1 and #2 in *L. sphaerica* (Table 1, Fig. 4).

Discussion

Since only three RAPD bands unique to *L. sphaerica* compared to eight bands unique to domesticated *L. siceraria* were found in the Zimbabwe collection, we conclude that this collection does not represent progeny of a recent cross between these two species. Nevertheless, the RAPDs indicate some similarity between the Zimbabwe collection and *L. sphaerica,* which may be the result of introgression or of randomly occurring band comigration (e.g., Reiseberg 1996). The occurrence of uniquely present or uniquely absent bands in the Zimbabwe samples could be in keeping with the hypothesis of introgression or could be evidence that the collection represents an evolutionary lineage distinct from both *L. sphaerica* and domesticated *L. siceraria.* Although non-parental bands are often found in hybrids, they typically represent fewer than 15% of the total number of bands in the hybrid (Huang et al. 2000). Given the much larger percentage (36%) of unique bands in the Zimbabwe collection, it seems likely that this collection represents a genetically distinct lineage that may or may not have hybridization in its history. However, we recognize that incomplete sampling could bear on this issue. For ex-

TABLE 1. SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs) IN THE TRN*R*-ATP*A* SPACER FOR ACCESSIONS OF *LAGENARIA*, INCLUDING THE WILD COLLECTION FORM ZIMBABWE. DATA FOR *L. SICERARIA* INCLUDES 16 COLLECTIONS FROM AFRICA, ASIA, AND THE AMERICAS. A = ADENINE, C = CYTOSINE, G = GUANINE, T = THYMINE.

Genetically distinct lineage	SNP #1	SNP #2	SNP #3	SNP #4	SNP #5
L. siceraria	C	G	A	G	A
wild Zimbabwe collection	A	G	A	G	A
L. sphaerica—South Africa (#1127)	C	G	C	T	G
L. sphaerica—South Africa (#1111)	A	G	C	T	G
L. sphaerica—Zimbabwe	A	T	C	T	G
L. breviflora	A	T	C	T	G
Cucurbita pepo	A	G	A	T	T

ample, a wider sampling of populations related to the Zimbabwe collection (assuming such populations exist) might reveal the presence of the four bands that were uniquely absent in the collection (Fig. 3). With respect to the bands that appeared to be uniquely present in the Zimbabwe collection, additional sampling of domesticated *L. siceraria* and *L. sphaerica*—species which have been shown to be genetically diverse here (Fig. 3, Table 1) and in Decker-Walters et al. (2001)—might reveal the presence of the Zimbabwe collection bands in these taxa. Consequently, it is difficult to be certain that all of the unique RAPD bands of the Zimbabwe collection are in fact unique to that collection.

The plastid sequence data were less ambiguous than the RAPD data. The SNP data revealed a distinct genetic profile for the Zimbabwe collection that differed from a uniform profile for

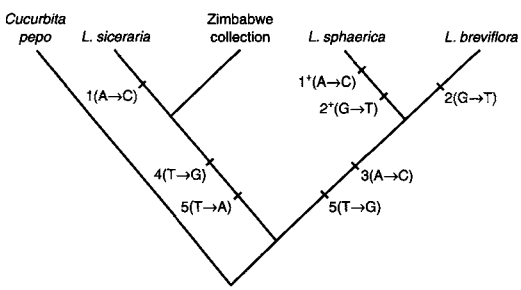

Fig. 4. The single most parsimonious phylogenetic tree based on the SNP data (Table 1) from the aligned sequences of the *trnR–atpA* spacer. For each of the five SNP sites, the nucleotide state change along a particular branch is given in parentheses after the site number. Character changes preceded by a '+' represent the addition of a second character state in a taxon. For example, *L. sphaerica* possessed both the ancestral base A and a derived base C at SNP site #1.

domesticated *L. siceraria* (Table 1). Thus, while it is clear from both the RAPD and SNP data that the Zimbabwe population is not merely an escape of cultivated bottle gourd, it is also unlikely that this population is closely related to the wild population pool that gave rise to domesticated bottle gourd. All of the sampled landraces of the domesticate, which represented the New World and Asia as well as Africa, shared base C at SNP site #1, while the Zimbabwe collection had the ancestral A at that site (Fig. 4). Therefore, if ocean currents or human transport dispersed wild bottle gourds from Africa to the New World and Asia before domestication (cf. Heiser 1973), then the wild source(s) would not have included populations with the more primitive genetic configuration seen in the Zimbabwe population. Alternately, these data could be used to support the hypothesis that this species was not dispersed to the New World before domestication, although this would require a scenario in which domesticated gourds somehow reached the Americas and were brought into use by the native peoples 9000–13 000 years ago in order to account for the early Peruvian (MacNeish et al. 1970) and Mexican (Cutler and Whitaker 1961) remains of *L. siceraria.* In any case, it is obvious that the Zimbabwe population represents a distinct and wild evolutionary lineage separate from that of domesticated bottle gourd.

Although the Zimbabwe population is genetically distinct, both in its RAPD profile and plastid sequence, its morphophysiology (including segregation for seed characteristics) indicates that it should be classified as *L. siceraria* and not as a new species. A full description and possibly new infraspecific classification of wild *L.*

siceraria must wait until a broader sampling can be obtained from Zimbabwe and possibly other parts of Africa. Further sampling of wild populations, particularly from northern Africa, which is the center of diversity for the genus, could provide more definitive evidence concerning where and from which wild lineage domestication of *L. siceraria* occurred.

Finally, RAPD (Fig. 3), SNP (Table 1), and seed (Fig. 2) variation in *L. sphaerica* were greater than expected, suggesting introgression with other species or perhaps the existence of two species in the *L. sphaerica* accessions examined herein. Additional sampling and marker assessment (particularly RAPDs) of geographically diverse populations of *L. sphaerica* are needed to determine the circumscription and range of genetic variation for this species, the extent of introgression with other species, and the possible existence of undescribed species of *Lagenaria.*

ACKNOWLEDGMENTS

This research was funded by the University of Wisconsin; The Cucurbit Network; U.S.D.A.; Conservation, Food & Health Foundation, Inc.; and the Regional Office for Southern Africa, The World Conservation Union (I.U.C.N.).

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